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Forum: Oxidative Stress Status

DETERMINATION OF SALICYLATE HYDROXYLATION PRODUCTS AS AN IN VIVO OXIDATIVE STRESS MARKER

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Abstract—The in vivo measurement of highly reactive free radicals, such as the ˙OH radical, is very difficult. New specific markers, which are based on the ability of ˙OH to attack the benzene rings of aromatic molecules, are currently under investigation . The produced hydroxylated compounds can be measured directly. In vivo, radical metabolism of salicylic acid produces two main hydroxylated derivatives (2,3- and 2,5-dihydroxybenzoic acids). The latter acid can be also produced by enzymatic pathways through the cytochrome P-450 system, while the former acid is reported to be solely formed by direct hydroxyl radical attack. Therefore, measurement of 2,3-DHBA, following oral administration of the drug acetyl salicylate, could be proposed for assessment of oxidative stress in vivo. In this paper, a sensitive method for the identification and quantification of hydroxylation products from the reaction of ˙OH with salicylate in vivo is presented. It employs a high performance liquid chromatography and electrochemical detection system. A detection limit of < 1 pmol for the hydroxylation products has been achieved with linear response over at least five orders of magnitude. Using this technique, we measured plasma levels of 2,3- and 2,5-DHBA dihydroxylated derivatives and salicylic acid and determined the ratios following administration of 1 g acetyl salicylate in 20 healthy subjects. © 2000 Elsevier Science Inc.

Keywords—Hydroxyl radical, Free radicals, Oxidative stress, Salicylic acid, Dihydroxybenzoates, HPLC-electrochemical detection, Humans

INTRODUCTION

Due to its high reactivity, the ˙OH radical has a very short half-life and is therefore present in extremely low concentrations. Some direct (electron spin resonance) or indirect (measurement of ethylene liberated from 2-keto 4-methylthiobutyrate, measurement of aromatic hydroxylation) methods have been proposed to probe the formation of ˙OH radical in vivo. ˙OH radicals are able to attack the benzene rings of aromatic molecules to produce hydroxylated compounds that can be measured directly. Aromatic hydroxylation has already been used for measuring in vitro ˙OH production [1–3]. If an aromatic compound can be safely administered to humans in doses that produce concentrations in body fluids sufficient to scavenge ˙OH, then assaying such products would be reasonable evidence that ˙OH is being formed in vivo, provided that these products are not formed by enzymatic hydroxylation. A suitable candidate for use in humans may be salicylate or its acetylated form.

O-acetyl salicylic acid (ASA) is a commonly used analgesic and anti-inflammatory agent in man. After ingestion, a substantial amount of ASA is hydrolyzed to

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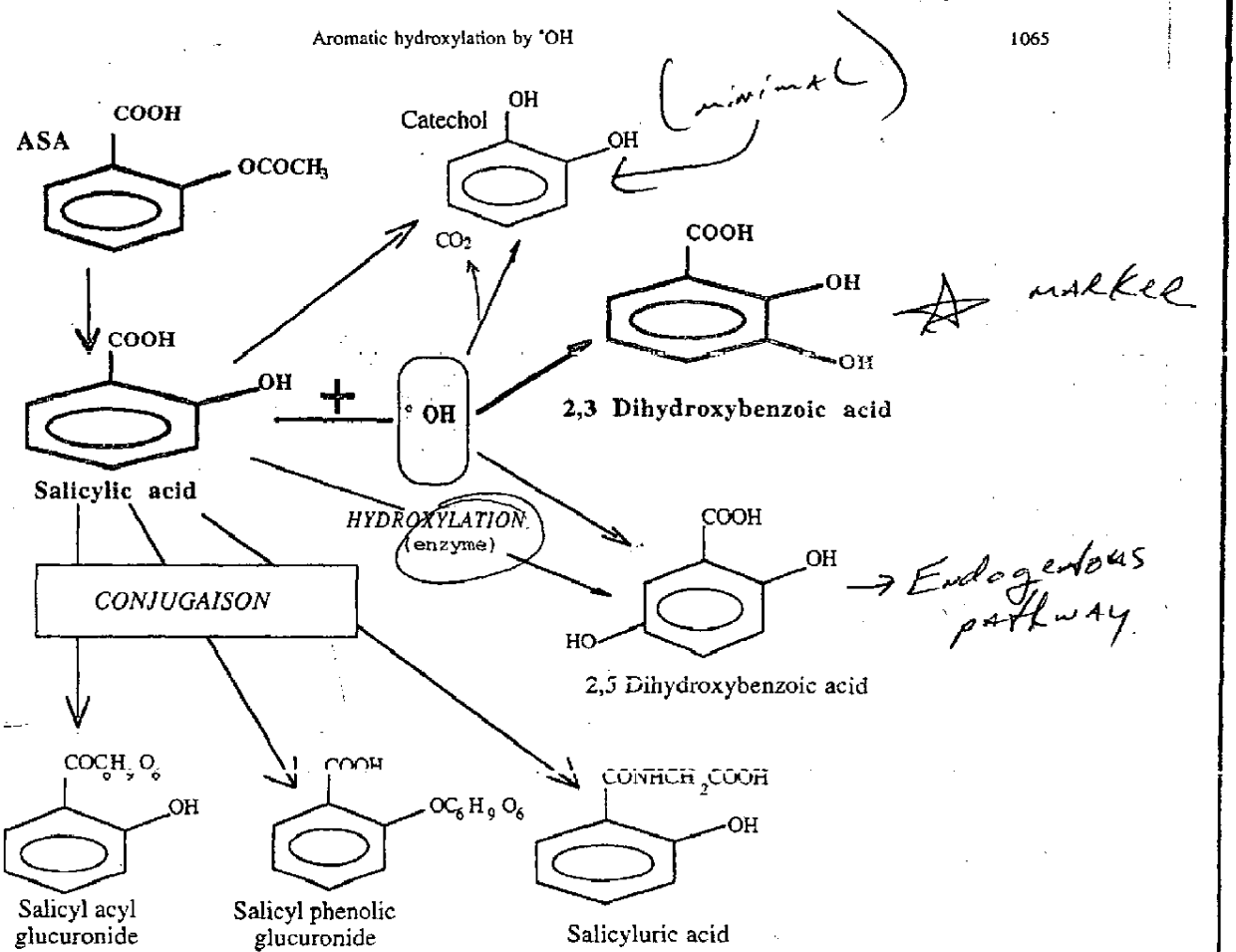

Fig. 1. Major reported enzymatic and oxidative by-products of ASA in human.

salicylic acid (SA) by esterases in the gastrointestinal tract, in the liver, and, to a smaller extent, in the serum [4] (Fig. 1). Salicylate reaches its peak in plasma about 0.5 to 1.5 h after the oral intake of ASA. SA is further metabolized by conjugation to glycine (liver glycine N-acetylase) to form salicyluric acid, by hydroxylation (liver microsomial hydroxylases) to form gentisic acid, and by formation of the phenolic glucuronide and the acyl glucuronide. About 60% of salicylate remains unmodified and can undergo °OH attack to produce two derivatives, namely 2,3-dihydroxybenzoate (2,3-DHBA) and, to a much smaller extent, catechol [5], that have not been reported as products of enzymatic metabolism. Thus 2,3-DHBA appears to be a useful marker of in vivo °OH production.

MEASUREMENT OF DIHYDROXYBENZOIC ACIDS

Chemicals

Sodium salicylate was obtained from Merck (Darmstadt, Germany); 2,3 dihydroxybenzoic acid, 2,5 dihydroxybenzoic acid, 2,6 dihydroxybenzoic acid, and 3,4 dihydroxybenzoic acid were purchased from Sigma (St. Louis, MO, USA). ASA (Aspégic) from Synthelabo, Le Plessis Robinson, France; Acetonitril, methanol, trisodium citrate, sodium acetate, ether, and ethyl acetate were obtained from Prolabo (Lyon, France). All chemicals were analytical reagent grade and used without further purification.

Instrumentation

A Hitachi Liquid Chromatograph pump equipped with an auto-sampler WISP (Waters) was used. Detection was performed with two electrochemical amperometric detectors (Bioanalytical Systems, model LC4B or Shimadzu L-ECD-6A) with plastic cells equipped with glassy carbon electrodes operated at +0.7 V, and an Ag/AgCl reference electrode. The signals from the detector were acquired on Varian DS601 data system and subsequently processed. The analytical stainless-steel column was 150 × 4,6 mm, and packed with octadecyl

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silane (Spherisorb ODS_2, C18) with average particle size of 3 μm (Alltech). A guard column packed with 10 μm spheri-10 PR18 (30 × 4.6 mm I.D., from Alltech) was also used. Peak areas at 0.7 V were recorded with a 1 mV potentiometric screen. All separations were performed at room temperature. For more information, see reference [6].

Optimization procedures

Choice of the oxidation potential. The hydrodynamic voltammograms from 0 to 1400 mV were recorded using 100 mg/l of all studied substances dissolved in the mobile phase (85% Phosphate buffer 100 mM, 0.10 mM SDS, pH 3.3, and 15% methanol, V/V). A CES detector (Erosep Instrument) equipped with 16 electrodes set up at variable potentials was used. We measured the signal/noise ratio of oxidation potential for the concerned substances, especially 2,3-DHBA. The 2,3-, 2,5-, and 3,4-DHBA acids produce a maximum current for a potential of about 0.7 V. However, at this voltage the other possible hydroxybenzoic acids 2,4-and 2,6-DHB acids, catechol, and salicylic acid do not yield a detector response. At a detector potential of 1.1 V, salicylic acid and all the dihydroxybenzoic acid products are recorded. An oxidation potential of 0.7 V vs. the Ag/AgCl reference electrode was chosen to obtain the maximum current response and minimum background noise for the hydroxylated products of salicylate.

Choice of the eluent. An isocratic delivery system was used consisting of a single eluent containing sodium acetate/trisodium citrate (30 mmol/30 mmol) pH 3,90. The flow rate was 0.2 ml/min. We found that addition of 15% of methanol in the mobile phase constitutes a good compromise with a retention time for SA of 7 min and 11 min for the internal standard 2,6 DHBA. The mobile phase was sparged continuously with helium gas during elution. We assayed different potential internal standards previously used by other investigators (3,4 DHBA, 2,4-DHBA, 2,6-DHBA, and catechol). The 2,4- and 2,6-DHBA do not yield a detector response at a detector potential set at 0.7 V, the 3,4-DHBA was thus used for the measurement of DHBAs. For SA assay, the 2,6 DHBA was found to be the most appropriate.

Extraction procedure. Extraction of plasma samples with ether or ethyl acetate were compared. DHBAs were better extracted with ethyl acetate. With double extraction, results were not different, and therefore one simple extraction with ethyl acetate was applied. The extraction with different volumes of ethyl acetate was then examined. We obtained satisfactory results with 3 ml of solvent when 0.4 ml of plasma sample was used.

The optimized procedure was as follows: Aliquots of standard solutions and of plasma samples (400 μl) were mixed with 100 μl of 2.5 μM 3,4-DHBA and acidified by 75 μl of concentrated HCl in 10 × 70 mm glass tubes. The samples were mixed on a vortex-type mixer for 30 s, 3 ml of ethyl acetate were then added and mixed for exactly 2 min. The tubes were finally centrifuged at 1600 × g for 15 min, and two ml of ethyl acetate phase were dried under nitrogen steam. The dry residue was redissolved in 200 μl of mobile phase and 100 μl were injected into the column. Mobile phase consisted of sodium citrate and acetate 30 mmol, pH 3.90. Flow rate was 0.2 ml/min. The concentrations for calibration curves were as follows: 25, 100, 400 nmol 2,3-DHB/l, and 125, 500, 2000 nmol 2,5-DHB/l, using peak-area ratios. Stock solutions of 2,3-DHBA, and 2,5-DHBA were stable for at least 2 months when stored at 4°C in the dark. Saline and plasma blanks were analyzed with each set of standards.

Analytical performance. The detection limit was found to be 0.37 nmol for 2,3-DHBA and 0.62 nmol for 2,5-DHBA. These low limits demonstrate the excellent sensitivity of the proposed method for both DHBAs measurement. The concentrations and peak areas showed linear relationships for 2,3- and 2,5-DHBA in the concentration range investigated (0 to 4000 and 5000 nmol/l, respectively). The absolute recoveries of 2,3-DHBA added at a concentration of 25 nmol/l in six different plasma samples ranged from 86 to 102% with a mean of 94%. Similar studies on 2,5-DHBA (250 nmol/l) yielded recoveries from 94 to 103% with a mean of 98%. The within-run coefficients of variation ranged from 3,3 to 4.1% for 2,3- DHBA and was of 2.1 for 2,5-DHBA. The between-run coefficients of variation ranged between 5.4 and 6.2% for 2.3 DHBA and from 5.7 to 7.4% for 2,5 DHBA. The between-run and recovery assays were conducted during a 15 d period.

Salicylic acid measurement

Salicylic acid assay was performed as previously described [7]. Briefly, aliquots (100 μl) of standard solutions or plasma samples were mixed with 100 μl of 2.5 μM 2,6-DHBA and deproteinized by 200 μl of ethanol in polypropylene conical tubes. The samples were mixed on a vortex-type mixer for exactly 2 min. The tubes were then centrifuged at 1600 × g for 15 min, and 50 μl of the supernatant was diluted with 950 μl of mobile phase. The diluted solution was then filtered on a 0.45 μ filter (Alltech) and 50 μl were injected into the column. The mobile phase consisted of sodium citrate and acetate 30 mmol/l, pH 5.45/methanol (85/15). The flow rate was 1

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ml/min and the detector was set up at 295 nm. Standard curves were constructed from measurements of peak-area ratios. The concentrations were as follows: 62.5, 250, 1000 μmol/l of SA.

The human study

To show the applicability of the proposed method, salicylic acid, 2,3-, and 2,5-DHBA concentrations were measured in 20 human subjects after a single oral dose of 1000 mg ASA (Aspégic) (1000 mg were dissolved in 150 ml of water, prepared just prior to administration). Blood samples were drawn before and 2 h following ASA administration. Plasma was separated, quickly frozen and stored at −20°C until required for analysis. Figure 2 shows a chromatogram of a blank plasma sample and a chromatogram of a plasma sample from the subject receiving ASA. Both 2,3- and 2,5-DHBAs were well separated and no interfering peaks were seen in blank plasma samples. Concentrations of 2,3- and 2,5-DHBA as low as 10 nmol/l could be accurately measured. Analysis of samples drawn before ASA administration shows that the plasma fractions were free of salicylic acid or its dihydroxybenzoic acids. The results were obtained from 20 healthy volunteers aged 20 to 40 years with a mean age of 28.2 ± 5.22 years (10 women and 10 men). Two h after ASA administration, the plasma SA level was 487 ± 116; 245–729 μmol/l, that of 2,3-DHBA was 65.2 ± 23.8; 13.6–113 nmol and that of 2,5-DHBA was 832 ± 309; 187–1477 nmol (M ± SD; M ± 2.086 SD).

DISCUSSION

In vivo metabolism of salicylic acid produces two main hydroxylated derivatives (2,3- and 2,5-DHBA) which could be measured. Salicylate reacts with ·OH at a rate constant of about $5 \times 10^{+9}$ to 10^{+10} $M^{-1}S^{-1}$ [8]. Low oral doses of ASA may give body-fluid salicylate concentrations, enough to trap ·OH radicals in vivo. It should be noted that the principle behind this methodology could be applied to other aromatic compounds such as phenylalanine or benzoic acid [9–11].

Many techniques have already been used to evaluate hydroxylated compounds in in vitro as well as in in vivo studies. A colorimetric method was initially described by studies [2,12] in which the hydroxylated phenols were treated with sodium tungstate and sodium nitrite in an acidic medium. The pink color was developed in an alkaline medium and measured with a spectrophotometer at 410 nm [13]. HPLC techniques were also developed to separate and quantify the hydroxylated derivatives in animals [1,14–15] or in humans [16–18]. Spectro-photometric and spectro-fluometric methods were adequate for vitro studies, where high concentrations of ·OH are usually formed; however, these detection modes are not sensitive enough to quantify hydroxylated derivatives in in vivo studies. The determination of hydroxyl radicals using salicylate as a trapping agent by gas chromatography-mass spectrometry has also been reported [19]. Consequently, the electrochemical detection mode became popular in most laboratories interested in the quantification of oxidant stress in vivo, especially those concerning human studies.

2,3-DHBA is usually considered a bona fide marker for detecting hydroxyl radicals. However, its level is markedly lower than that of 2,5-DHBA [14]. Our findings confirm a significantly higher level of 2,5-DHBA than 2,3-DHBA (3- to-5-fold). The selective difference between 2,3- and 2,5-DHBA could be associated with differing metabolic rates of production of these adducts, different efficiencies of scavenging ·OH radicals at the 3 or the 5 position of the salicylate aromatic ring, and differences in the stability of the adducts against metabolic or biochemical modifications by cellular components [20]. In addition, effect of plasma binding of these adducts on their distribution in blood and into lymph, their transformation and their excretion in the urine has been reported [21]. However, this difference seems to be due to the fact that 2,5-DHBA, but not 2,3-DHBA, is produced by an enzymatic hydroxylation pathway through the cytochrome P-450 microsomal system [22]. The latter seems to be formed exclusively via a radical pathway, which further substantiates the validity of the assumption that 2,3-DHBA authentically reports free radical fluxes in vivo. However, in a cautionary note, Halliwell et al. [23] stressed that 2,3-DHBA derivative may be generated by an unreported minor metabolic pathway, which may be discovered in the future.

The presence of 2,3-DHBA in the plasma of healthy subjects after ASA intake could be due to the baseline rate of intracellular ·OH formation from ionizing radiation and the Fenton reactions in vivo [5]. This phenomenon is supported by the detection of baseline values of several markers of ·OH production such as allantoin, 8-hydroxy-deoxyguanosine, or thymidine glycols in plasma or urine of healthy subjects.

The 2,3-DHBA derivative appears to be a sensitive and specific marker of in vivo oxidative stress studies. There is now increasing evidence for 2,3-DHBA production after ASA administration in conditions of oxidative stress such as exposure to 100% oxygen, treatment with the redox-cycling drug Adriamycin [14], in vitro neutrophiles activation [24], diabetes [17], alcoholism [25], or rheumatoid arthritis [5].

Because the plasma level of 2,3-DHBA depends on that of salicylate, and because the latter could vary among individuals ingesting the same dose of ASA, the

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Fig. 2. HPLC-electrochemical detection chromatogram showing separation of isomeric DHBAs. (A) Elution of a standard mixture containing 125 nmol/l 2,3-DHBA, 1000 nmol/l 2,5-DHBA and 500 nmol/l of internal standard 3,4-DHBA. (B) and (C) Plasma samples from a normal subject before and after 1 g per os ASA administration, respectively. The identification of peaks is as follows: (1) 2,5-DHBA; (2) 2,3-DHBA; and (3) 3,4-DHBA.

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plasma concentration of 2,3-DHBA should be expressed as 2,3-DHBA/salicylate ratio (mmoles/moles). To overcome such a disadvantage, an alternative possibility may be the intravenous administration of SA. Such alternative has been used in rats and cats [26–27]. This possibility deserves the attention of workers in this field in humans, where nothing has yet been done.

SOME PRECAUTIONS

Salicylate can be hydrolyzed on addition of peroxynitrite, and it is uncertain whether or not ·OH is responsible. Thus, caution should be exercised using this method in systems where $ONOO^-$ is being formed [28–29].

It should be noted that plasma ASA is rapidly hydrolyzed (40% hydrolysis in 120 min at room temperature after sampling) and this could increase plasma level of salicylic acid [30]. Indeed, this hydrolysis is faster in whole blood than in plasma, and is attributed to plasma and red blood cell esterases. It is thus necessary to remove red blood cells from plasma as rapidly as possible. However, it appears that plasma enzymatic hydrolysis is completely inhibited by physostigmine (200 μmol). This should prevent an increase in salicylate plasma level after blood was drawn. In our study, plasma ASA level, 2 h after ASA administration should be negligible. However, at higher doses of ASA or when samples are drawn in the early periods, inhibition of esterases should be considered.

Particular attention should be paid to avoid hydroxylation of aromatic compounds by free transition metals during measurement. Transition metal-free, adequately washed tubes and high pure water should be used throughout the assay. This is especially true for in vitro or ex vivo studies (dialysate, perfusate,) were no chelating agents to neutralize free transition metals. Several teams are working in this field [31–32]. We had some difficulties obtaining reproducible results when we attempted to determine the production of radical hydroxyl in rats after ischemia reperfusion sequence (nonpublished data). Montgomery et al. [33] noted that artifactual production of dihydroxy-benzoates may occur by components of the experimental apparatus of intra-cerebral micro-dialysis system.

FURTHER RESEARCH

Many aspects still remain to be investigated.

1) Larger doses of ASA should be examined. In fact, in humans, ASA is generally not acutely toxic until the plasma concentration of salicylic acid reaches the millimolar range. Thus, the ASA dose used in the present study was well below the toxic concentrations.
2) Salicylic acid has less pharmacological effects than ASA. For example, inhibition of platelet function by ASA could underestimate ·OH production by the cyclooxygenase pathway. The direct administration of sodium salicylate could overcome this concern.
3) Because 2,3- and 2,5-DHBA could be metabolized and excreted in urine, their determination in urine may be of great interest.
4) Evaluation of the relationships between 2,3-DHBA levels and the antioxidant defense systems in different oxidative stress should now be undertaken.

CONCLUSION

Direct evidence for the formation of free radicals in oxidative processes and the causal relationship between free radicals and damage are still lacking. Despite the emergence of electron spin resonance and spin trapping techniques, the identification and the characterization of oxygen-derived free radicals in humans still remains a formidable task. The salicylate hydroxylation products assay as described here provides a simple and convenient method by which ·OH radicals may be detected and quantified in vivo. It is hoped that this methodology will be useful for those attempting to detect and measure ·OH generation in oxidative diseases such as diabetes, myocardial infarction, rheumatoid arthritis, cancer, and aging.

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ABBREVIATIONS

ASA—acetyl salicylic acid
2,3-DHBA—2,3-dihydroxybenzoic acid
2,5-DHBA—2,5-dihydroxybenzoic acid
2,6-DHBA—2,6-dihydroxybenzoic acid
3,4-DHBA—3,4-dihydroxybenzoic acid
ECD—electrochemical detection
H_2O_2—hydrogen peroxide
HPLC—high performance liquid chromatography
DHBAS—dihydroxybenzoic acids
$^{\circ}O2$—superoxide anion radical
$^{\cdot}OH$—hydroxyl radical
ROS—reactive oxygen species
SA—salicylic acid

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